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DEVELOPMENTAL BIOLOGY

Developmental Biology 264 (2003) 240–254

www.elsevier.com/locate/ydbio

Math5 determines the competence state of retinal ganglion cell progenitors

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Received for publication 30 May 2003, revised 29 July 2003, accepted 6 August 2003

Abstract

In mice, all of the six retinal neuron types are generated from common multipotent retinal progenitors, and their differentiation from progenitors is regulated by both extrinsic and intrinsic factors. Previously, we showed that targeted deletion of the *atonal (ato)* homologue *math5* blocked the differentiation of most retinal ganglion cells (RGCs), revealing an essential role for *math5* in RGC differentiation. In this study, we used the Cre-loxP recombination system to trace the fate of *math5*-expressing cells in retina. Our results demonstrated that *math5* expression was associated with the differentiation of multiple retinal neuron types, including RGCs, photoreceptor, horizontal, and amacrine cells, implying that *math5* expression alone is not sufficient to determine the RGC fate. Math5 expression was restricted to postmitotic cells in developing retina, suggesting that cell fate commitment of retinal neurons occurs after the terminal mitosis. The insufficiency of and requirement for *math5* in RGC differentiation indicates that, like *ato* in the development of *Drosophila* R8 photoreceptors, *math5* plays a role in determining the RGC competence state of retinal progenitors and that additional positive and negative factors are required in determining RGC fate. Furthermore, we show that loss of Math5 function severely reduced the RGC expression of the transcription factors Brn-3b, Gfi-1, Isl-1, Isl-2, Nscl-1, Nscl-2, and RPF-1, suggesting that Math5 expression is required to activate a comprehensive transcription network of RGC differentiation.

Keywords: *math5*; Retina; Cell lineage; Neurogenesis; Retinal ganglion cells; Basic helix–loop–helix (bHLH)

Introduction

Due to its simplicity in neuronal composition, easy accessibility, and unique laminar structure, the mammalian retina serves as an excellent model to study the central nervous system (CNS) development. Birthdating experiments using ^{3}H-thymidine labeling demonstrated that the formation of retinal neurons follows a phylogenetically conserved order (Cepko et al., 1996). RGCs are always the first born neurons. Cones, horizontal cells, and amacrine cells are born at about the same time as, though not before, RGCs. Rod photoreceptors and bipolar cells are born last. Retroviral infection and cell lineage tracer injection of retinal progenitors in mammals and amphibia have shown that six retinal cell types are generated from a common pool of multipotent progenitors and that each progenitor can produce clones with one to six cell types, suggesting that the multipotency of progenitors can be maintained until the final cell division (Holt et al., 1988; Turner and Cepko, 1987; Wetts and Fraser, 1988). Recent studies have shown that the comprehensive processes of retinal differentiation are regulated by both extrinsic and intrinsic factors. While embryonic progenitors taken from the period of peak ganglion cell formation are competent to differentiate into RGCs, progenitors from developing stages when the retina is not normally generating RGCs do not (Austin et al., 1995). Conversely, while late progenitors from periods of rod cell genesis can readily differentiate into rods in vitro, early embryonic progenitors have a lower rate of rod differentiation (Watanabe and Raff, 1990). In addition to in-

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doi:10.1016/j.ydbio.2003.08.005

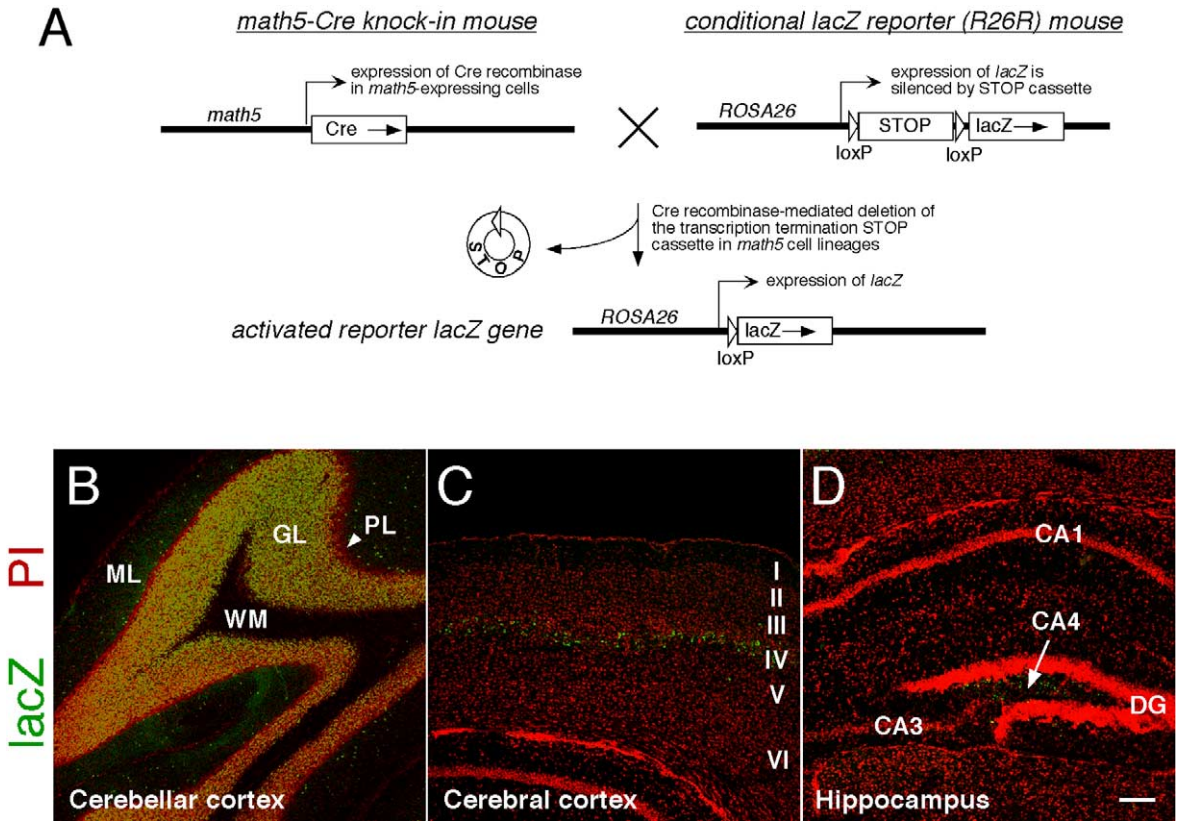

Fig. 1. Lineage tracing of *math5*-expressing cells with the Cre-loxP recombination system. (A) The Cre-loxP system for conditional activation of reporter *lacZ* expression using *math5*-Cre and R26R-*lacZ* mice. (B–D) Tissues from double heterozygous $math5^{Cre/+}$ $R26R^{lacZ/+}$ mice were sectioned and analyzed for the expression of β-galactosidase. Cells of *math5* origin are detected by anti-β-galactosidase (green). Sections were counterstained with PI (propidium iodine, red). (B) Section of cerebellar cortex. ML, the molecular layer; PL, the Purkinje cell layer; GL, the granular layer; WM, white matter. (C) Section of cerebral cortex. I, the molecular layer; II, the external granular layer; III, the external pyramidal layer; IV, the internal granular layer; V, the internal pyramidal layer; VI, the multiform layer. (D) Section of hippocampus. CA1-4, cornus ammonis division 1–4; DG, dentate gyrus. Scale bar, 100 μm.

trinsic properties, previous studies have indicated that the generation of retinal neuron cell types is regulated by extrinsic factors. The Notch-Delta pathway has been shown to impact the cell fate choice of RGCs as well as other retinal neurons (Austin et al., 1995; Bao and Cepko, 1997; Dorsky et al., 1995). Results from reaggregate culture experiments of retinal cells indicate that progenitors' choice of amacrine and cone cell fates is influenced by unidentified extrinsic factors (Belliveau and Cepko, 1999). Furthermore, a number of diffusible molecules are known to affect the differentiation of late-born retinal cell types. For example, taurine and 9-*cis* retinoic acid act to promote the rod fate choice (Altshuler et al., 1993; Kelley et al., 1994), CTNF, the bipolar cells (Ezzeddine et al., 1997), and EGF, the glial cell fate (Lillien, 1995).

To explain the birth order and cell fate choices of progenitors, a competence model of retinal neurogenesis has been proposed (Cepko et al., 1996; Livesey and Cepko, 2001). In this model, retinal progenitors go through a series of competence states during embryogenesis. At each competence state, progenitors are competent to differentiate into a specific subset of retinal neurons. The competence states of progenitors are determined intrinsically and the generation of specific neuronal cell types is regulated by positive and negative environmental or extrinsic factors. Therefore, at a given developmental stage, the capability of a retinal progenitor to differentiate into a specific retinal neuron is determined intrinsically by the expression of a specific subset of regulatory genes and by the retinal environment at that defined stage. However, the genes that determine the intrinsic properties and the differentiation competence of retinal progenitors are poorly understood.

Basic helix–loop–helix (bHLH) transcription factors play crucial roles in cell fate determination and differentiation during retinal development (Cepko, 1999; Hsiung and Moses, 2002; Kageyama et al., 1995). In *Drosophila melanogaster*, *ato* initially acts as a proneural gene and the expression of *ato* endows cells with neural competence (Jarman et al., 1994). During the development of *Drosophila* retina, *ato* is required for the specification of neuronal identity of the first born retinal neuron, the R8 photoreceptor cell (Brunet and Ghysen, 1999). Recent studies have demonstrated that many of the *atonal*- and *achaete-scute*-class bHLH transcription factors are expressed in the developing vertebrate retina. For example, *math3* and *NeuroD* (*atonal*-class factors) have been shown to regulate the cell differ-

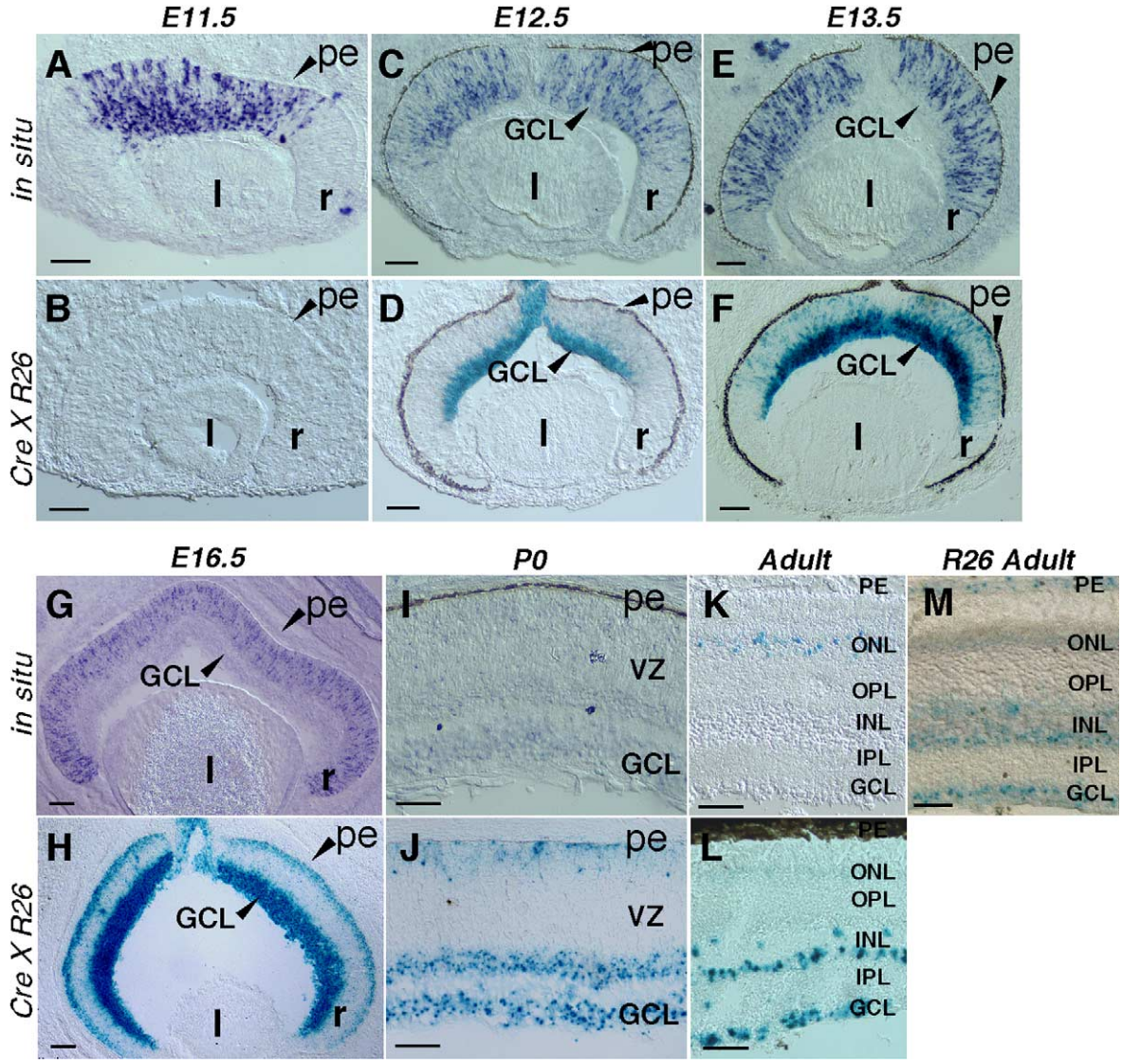

Fig. 2. Expression of *math5* and R26R-*lacZ* in developing retinas. In situ hybridization of sectioned wild type retinas with *math5* riboprobes at different developmental stages (A, C, E, G, and I). (K) X-Gal staining of an adult heterozygous $math5^{lacZ/+}$ retina. (B, D, F, H, J, and L) Corresponding X-Gal staining of double heterozygous $math5^{Cre/+}$ $R26R^{lacZ/+}$ retinas at indicated developmental stages. (M) X-Gal staining of ROSA26 adult retina control. r, retina; l, lens; pe, pigmented epithelium; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar, in (A–H) and (M), 100 μm; in (I–I), 50 μm.

entiation of amacrine cells, and targeted deletion of *math3* and *NeuroD* results in the absence of amacrine cells (Inoue et al., 2002b). Other transcription factors have also been implicated in the differentiation of retinal neurons along with bHLH factors. The development of retinal photoreceptors has been shown to be regulated by *NeuroD* and *Crx* (a homeobox factor) (Furukawa et al., 1997; Morrow et al., 1999). *math3*, *mash1* (an *achaete-scute*-class factor), and *Chx10* (a homeobox factor) regulate the formation of bipolar cells (Burmeister et al., 1996; Hatakeyama et al., 2001; Tomita et al., 2000). These studies indicate that both bHLH and homeobox transcription factors likely act as the intrinsic factors to regulate the specification of retinal neuronal cell types.

In vertebrate retina, RGCs are the only output neurons that project axons outside the eye. Previous studies have shown that the POU-domain transcription factors Brn-3b and Brn-3c play pivotal roles in the differentiation processes of RGCs (Turner et al., 1994; Wang et al., 2002; Xiang et al., 1993). Targeted mutation in *brn-3b* leads to the loss of 70% RGCs (Erkman et al., 1996; Gan et al., 1996). Cell lineage analyses using *brn-3b-lacZ* (β-galactosidase) and *brn-3b-AP* (human placental alkaline phosphatase) reporter genes in knock-in mice have demonstrated that *brn-3b* is essential for axon growth, pathfinding, fasciculation, and survival of RGCs (Gan et al., 1999; Wang et al., 2000). However, *brn-3b* is unlikely to determine RGC competence of retinal progenitors because the initial differentiation and migration of RGCs is unaffected in *brn-3b*-null retina. Recently, studies have shown that the differentiation of RGCs

in mice coincides with the onset of *math5* expression and that targeted deletion of *math5* leads to the loss of most RGCs, implying an essential role for *math5* in RGC development (Brown et al., 2001; Wang et al., 2001). Furthermore, loss of Math5 function results in the absence of RGC differentiation and the abolished expression of *brn-3b*, suggesting that *math5* and *brn-3b* constitute an essential regulatory pathway for RGC differentiation and that *math5* acts upstream of *brn-3b* to activate the comprehensive RGC differentiation process.

So far, targeted deletion experiments have only demonstrated the requirement for *math5* in the differentiation of RGCs because RGCs are mostly absent in *math5*-null retina. The molecular mechanisms of *math5* function in RGC differentiation remain elusive. In this report, we test the precise role of Math5 in the development of mouse retinal neurons by lineage analysis of *math5*-expressing cells using Cre-loxP recombination system (Tsien et al., 1996) with *math5-Cre* and conditional reporter *R26R-lacZ* mice. We demonstrate that the expression of *math5* alone is insufficient to direct the differentiation of RGCs during normal retinal development. Rather, *math5*-expressing cells give rise to multiple retinal cell types, including RGCs, amacrine, horizontal, and photoreceptor cells. Furthermore, we show that *math5* expression is only detected in postmitotic retinal cells, suggesting that retinal cell fate is determined after cell cycle exit. The absence of RGC formation in *math5* knockout mice and the insufficiency of *math5* to determine RGC fate indicate that Math5 plays a role in determining the RGC competence of retinal progenitors and that Math5 is essential for the activation of a network of transcription factors in developing RGCs, including Brn-3b, RPF-1, Isl-1, Isl-2, Nscl-1, and Nscl-2.

Materials and methods

Animals

The *R26R-lacZ* conditional *lacZ* reporter and *ROSA26-lacZ* constitutive *lacZ* reporter mice were obtained commercially from Jackson Laboratory (Stock Number 003310 and 002292, respectively) and PCR genotyping of the reporter mice was performed according to protocols provided by Jackson Laboratory. *math5* and *brn-3b* mutant mice were generated as previously described (Gan et al., 1999; Wang et al., 2001). The *math5-Cre* knock-in construct was generated in a similar way to the original *math5-lacZ* knock-in construct (Wang et al., 2001). The *math5* coding region was replaced with Cre recombinase coding region from *Cre*-containing plasmid pBS185 (Invitrogen). The construct fuses *Cre* immediately upstream of *math5* translation initialization codon in the 5′ untranslated region and places *Cre* under the transcription control of *math5* regulatory sequences. To generate *math5*-Cre knock-in mice, the *Not*I-linearized *math5-Cre* construct was electroporated into AB1 embryonic stem cells, and three targeted clones were obtained by drug selection with G418 and FIAU and Southern blotting genotyping. Two targeted ES cell clones were injected into C57BL/6J blastocysts to generate mouse chimeras and the generation of heterozygous *math5-Cre* mutant mice was done as previously described in 129SvEv and C57BL/6J mixed background (Gan et al., 1996, 1999). Embryos were considered as E0.5 at noon on the day at which vaginal plugs were observed.

Immunohistochemistry, in situ hybridization, and X-gal staining

Working dilutions and sources of the following antibodies were used in this study: mouse anti-BrdU (1:200, Becton Dickson), mouse anti-Brn-3a (1:100, Santa Cruz), goat anti-Brn-3b (1:2,000, Santa Cruz), mouse anti-calbindin (1:5,000, Sigma), sheep anti-Chx10 (1:200, Exalpha), mouse anti-β-galactosidase (*lacZ*) (1:500, Developmental Studies Hybridoma Bank, DSHB), rabbit anti-β-galactosidase (1:500, Chemicon), mouse anti-Isl-1/2 (1:400, DSHB), rabbit anti-Phospho-Histone 3 (1:400, Santa Cruz), and mouse anti-Pax6 (1:200, DSHB). Alexa-conjugated secondary antibodies were obtained from Molecular Probes and were used at a concentration of 1:400.

Embryo and tissue samples were dissected and immediately fixed in 4% paraformaldehyde. The samples were then embedded and frozen in OCT medium (Tissue-Tek). Sections were cut at 20 μm, and section in situ hybridization analysis was performed as described (Li and Joyner, 2001). The anti-sense RNA probes used in this study were generated by using the following cDNA sequences: *math5* (mouse E14.5 retinal EST clone #2104–46) (Mu et al., 2001), *brn-3b* (#6063–67), *RPF1* (#0053–26), *Isl-1* (#6033–80), *Isl-2* (#8071–27), *Nscl-2* (#0252–95), *Nscl-1* (Brown et al., 1992), *Gfi1* (Gilks et al., 1993), *Gfi1b* (Tong et al., 1998).

Detection of β-galactosidase activities by X-Gal staining was done as previously described (Gan et al., 1999). Briefly, retinal tissues were fixed in 4% paraformaldehyde in PBS at 4°C for 30 min. Frozen sections were prepared at 16 μm and stained overnight at room temperature with 0.1% X-Gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$ in PBS.

BrdU labeling

For bromodeoxyuridine (BrdU, Sigma) labeling experiments, pregnant females were injected intraperitoneally with 100 μg BrdU/gram body weight 1 h before they were sacrificed. Embryo processing and BrdU labeling were performed as previously described (Mishina et al., 1995).

Results

Activation of reporter lacZ expression in math5 cell lineages

Previous studies have shown that, in the absence of *math5*, RGCs fail to form during retinal neurogenesis and that an increased production of cone and amacrine cells is detected, suggesting that *math5* plays a role in regulating the cell fate choice of retinal progenitors (Brown et al., 2001; Wang et al., 2001). The transient expression pattern of *math5* in the ventricular zone of the developing retina and the absence of *math5* expression in the ganglion cell layer (GCL) also indicate its early role in retinal progenitors. To further define the function of *math5*, we generated *math5-Cre* knock-in mice to investigate the fate of *math5*-expressing cells. Similar to our previously described *math5-lacZ* and *math5*-GFP knock-in mice (Wang et al., 2001), the $math5^{Cre/+}$ heterozygotes were viable and phenotypically indistinguishable from wild type littermates. No retinal defect was detected in heterozygous $math5^{Cre/+}$ mice, and homozygous $math5^{Cre/Cre}$ mutant mice showed a severe loss of RGCs comparable to those of $math5^{lacZ/lacZ}$ and $math5^{GFP/GFP}$ mice (data not shown).

Due to the transient property of *math5* expression, it was not possible to follow the fate of *math5*-expressing cells by the expression of *math5*. The Cre-loxP recombination system provides an effective approach to specifically activate the constitutive expression of *lacZ* gene as a cell lineage marker in the progeny of *math5*-expressing cells. The *math5-Cre* knock-in mice express Cre recombinase under *math5* regulatory sequences. By crossing *math5-Cre* mice with the conditional *lacZ* reporter *R26R-lacZ* mice in which cells constitutively express β-galactosidase activity after a Cre-mediated recombination event (Soriano, 1999), the cell fate of *math5*-expressing cells could be followed and characterized in the adult retina (Fig. 1A). Previous in situ hybridization studies have shown that *math5* is expressed in the developing retina and the developing tenth cranial ganglion (Brown et al., 1998; Wang et al., 2001). Due to the limitations of in situ hybridization, the complete temporal and spatial expression of *math5* has not been analyzed. The conditional expression of *lacZ* in double heterozygous $math5^{Cre/+}$ $R26R^{lacZ/+}$ mice could serve as a useful tool to investigate the expression profile of *math5*, particularly in cells that express it transiently during development. As shown in Fig. 2B–D, X-Gal staining of $math5^{Cre/+}$ $R26R^{lacZ/+}$ mice revealed that, in addition to retina and the tenth cranial ganglion, β-galactosidase activities were also detected in cells of the cerebellar cortex, cerebral cortex, and hippocampus. In the cerebellar cortex, *lacZ* expression indicated that cells of *math5* origin gave rise to granular cells in the tightly packed granular cell layer and in cells in the molecular layer, but not Purkinje cells (Fig. 1B). In the cerebral cortex, cells derived from *math5* lineages were detected in the internal granular cell layer (Layer IV) composed of closely packed stellate cells (Fig. 1C) and in cells of the CA4 (hilus) region of the hippocampus (Fig. 1D). The detection of β-galactosidase-positive cells suggests an involvement of *math5* in CNS development. However, the precise role of *math5* in CNS development has yet to be determined.

In the mouse retina, the expression of *math5* first appears at E11, and its expression expands circumferentially from E11–E16.5 (Brown et al., 1998; Wang et al., 2001). As shown in Fig. 2A and C, the expression of *math5* was distributed throughout the retina at E12.5. At E13.5 and later, its expression was restricted to cells in the ventricular zone and in the ciliary margin of the developing retina and was excluded from the newly formed GCL (Fig. 2E and G). From birth (P0) to adult, weak expression of *math5* in retina could only be detected in photoreceptor cells in the outer nuclear layer (ONL) (Fig. 2I, and data not shown). In contrast to the expression of *math5* mRNA in the ventricular zone at E12.5–E13.5, β-galactosidase activity in the $math5^{Cre/+}$ $R26R^{lacZ/+}$ retina was largely restricted to the newly formed GCL as well as in cells of the ventricular zone (Fig. 2D and F). At E16.5, the majority of β-galactosidase activity was confined to the GCL and the outer boundary of the ventricular zone (Fig. 2H). At P0, the expression of *lacZ* was detected in three defined layers: the GCL, the outer and inner boundaries of the ventricular zone (Fig. 2J), which contrasts to the diminished *math5* expression shown by in situ hybridization (Fig. 2I), and by X-Gal staining of the *math5-lacZ* knock-in reporter (Wang et al., 2001). In the adult retina, compared with *math5* expression as detected by the *math5-lacZ* knock-in reporter in photoreceptor cells in the ONL (Fig. 2K), the expression of *lacZ* was found in cells in the GCL, the inner and outer boundaries of the INL, as well as weakly in the ONL (Fig. 2L). The β-galactosidase activity in the adult $math5^{Cre/+}$ $R26R^{lacZ/+}$ retina closely resembled that in control ROSA26-*lacZ* retina (Fig. 2M). Compared with the onset of *math5* mRNA expression at E11 (Brown et al., 1998) and with the strong expression of *lacZ* throughout the E11.5 retina under a constitutive promoter in ROSA26-*lacZ* mice (data not shown), the R26R-*lacZ* reporter expression at E12.5 suggested an approximate 1-day delay of *lacZ* expression in $math5^{Cre/+}$ $R26R^{lacZ/+}$ mice. Such a delay was likely due to the requirement for the Cre recombinase-mediated deletion of transcription termination sequences preceding the β-galactosidase coding region. The difference between *math5* mRNA accumulation and *lacZ* expression also indicates the relatively short life of *math5* mRNA in early retinal cells and the constitutive expression of *lacZ* in the progeny of *math5*-expressing cells. Thus, the conditionally activated *lacZ* expression serves as a useful marker to study *math5* cell lineages.

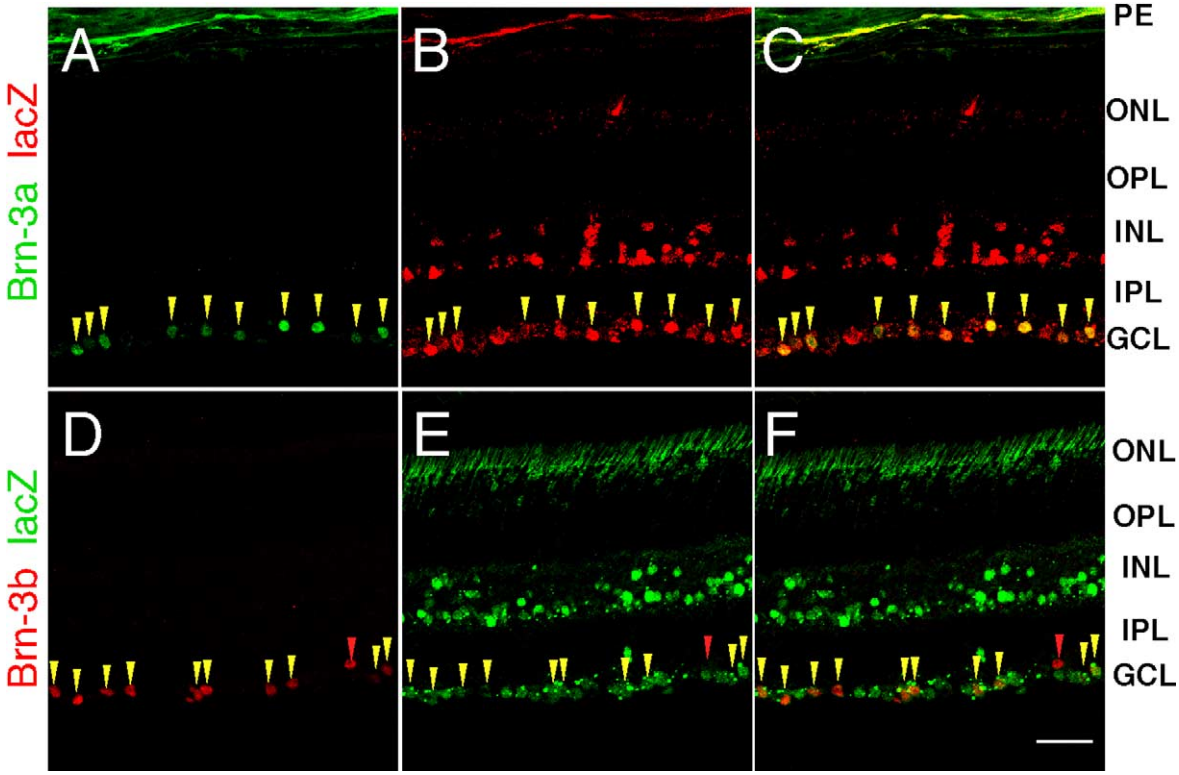

Fig. 3. Retinal ganglion cell fate of *math5*-expressing cells. The adult double heterozygous *math5*$^{Cre/+}$ *R26R*$^{lacZ/+}$ retinal sections were prepared for double immuno labeling with anti-β-galactosidase and RGC markers. (A) Anti-Brn-3a (green) labeling identifies the nuclei of RGCs in the GCL. (B) Anti-β-galactosidase (red) labels the cells of *math5* origin. (C) Merged image of (A) and (B). (E) Anti-Brn-3b (red) shows the nuclei of RGCs in the GCL. (E) Anti-β-galactosidase (green) labels the cells of *math5* origin. (F) Merged image of (D) and (E). Yellow arrowheads, colocalization of RGCs with anti-β-galactosidase. Red arrowhead, RGC from a non-*math5* origin. PE, pigmented epithelium; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar, 50 μm.

Expression of math5 in multiple retinal neuron cell lineages and the competence role of math5 in retinal development

The decreased number of RGCs and the accompanied increase of cone and amacrine cells in the *math5*-null retina suggest that *math5* plays a vital role in regulating the cell fate choice of retinal progenitors. Nevertheless, it remains unknown whether *math5* is expressed in non-RGC neurons during normal retinal development and plays a direct role in their differentiation. The targeted activation of *lacZ* in *math5* cell lineages provides a powerful tool to address these questions. As is shown in Fig. 2L, in adult retina, four rows of *math5* lineage cells were distributed in the GCL, the ONL, and the outer and inner boundaries of the INL. To characterize the neuronal cell types of these cells, we carried out double immunolabeling experiments of adult *math5*$^{Cre/+}$ *R26R*$^{lacZ/+}$ retinas with anti-β-galactosidase and specific cellular markers for retinal cells. The POU-domain transcription factors Brn-3a and Brn-3b are expressed in a majority of RGCs and serve as markers to identify RGCs (Xiang et al., 1995). As shown in Fig. 3A and D, immunostaining of retina sections with anti-Brn-3a and anti-Brn-3b showed the specific nuclear labeling of RGCs in the GCL. Labeling with an anti-β-galactosidase antibody revealed four rows of *math5* lineage cells in the retina (Fig. 3B and E). Double immunostaining with anti-β-galactosidase and anti-Brn-3a or anti-Brn-3b antibodies showed that nearly all Brn-3a- and Brn-3b-positive RGCs expressed β-galactosidase (Fig. 3C and F) with occasional detection of Brn-3-positive and β-galactosidase-negative RGCs (red arrow, Fig. 3F). Our results further confirmed that most RGCs are derived from *math5* lineages and that their formation depends on the presence of Math5 during development. The remaining RGCs are generated through *math5*-independent mechanisms and also express RGC markers, such as Brn-3a and Brn-3b. As shown in Fig. 3, not all of the β-galactosidase-positive cells in the GCL are Brn-3-positive. In mice, the GCL consists of approximately equal numbers of RGCs and displaced amacrine cells (Barnstable and Drager, 1984; Perry, 1981). Thus, the Brn-3-negative and β-galactosidase-positive cells in the GCL are likely the displaced amacrine cells.

The presence of β-galactosidase activity in the ONL suggests that *math5*-expressing cells gave rise to photoreceptors. β-Galactosidase-positive cells in the INL were detected in the outer and inner boundaries of the INL, suggesting their identities as horizontal and amacrine cells,

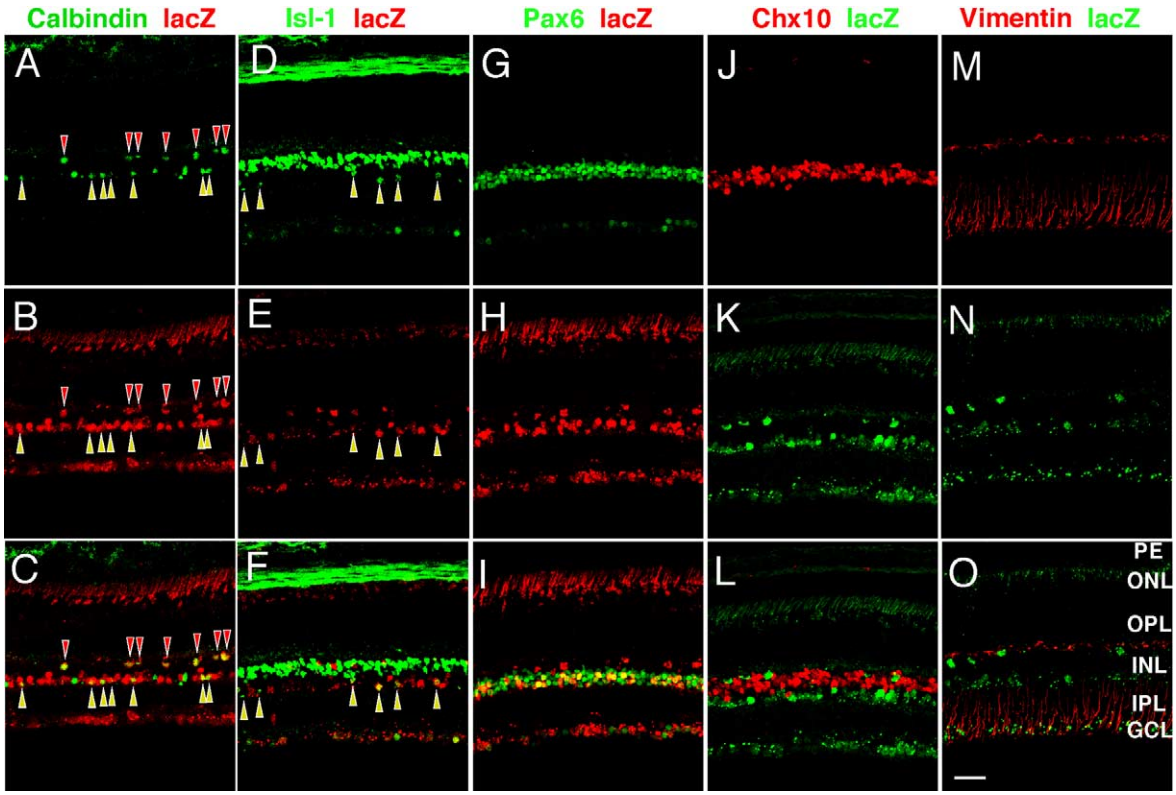

Fig. 4. Expression of *math5* in non-RGC cell lineages. Double immunolabeling of $math5^{Cre/+}$ $R26R^{lacZ/+}$ retinal sections with anti-β-galactosidase and retinal cell markers shows the presence of β-galactosidase in amacrine and horizontal cells but not in bipolar and glial cells. (A–C) Anti-calbindin (green) and anti-β-galactosidase (red) labeling identifies horizontal cells (red arrowheads) at the outer boundary and amacrine cells (yellow arrowheads) at inner boundary of the INL. Anti-Isl-1 (D-F) and anti-Pax6 (G-I) show amacrine cells of *math5* origin. Anti-Isl-1 (D–F) and anti-Chx10 (J–L) show no colocalization of bipolar cells with *math5* cell lineage in the INL. (M–O) Anti-vimentin reveals no co-localization in glial cells. PE, pigmented epithelium; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar, 50 μm.

respectively. To confirm their identities, we examined the β-galactosidase-positive cells with specific markers: calbindin for amacrine and horizontal cells (Inoue et al., 2002a), Isl-1 for bipolar cells in the outer half of the INL, cholinergenic amacrine, and ganglion cells (Galli-Resta et al., 1997), Chx10 for rod bipolar cells throughout the INL (Chow et al., 2001), and Pax6 for amacrine and ganglion cells (Marquardt et al., 2001). As shown in Fig. 4A–C, all calbindin-positive cells in the outer boundary of the INL (red arrowheads) coexpressed β-galactosidase, suggesting that all horizontal cells are derived from a *math5* lineage. Similarly, amacrine cells originated from Math5 lineage cells were shown by colocalization of β-galactosidase with calbindin and Isl-1 in the inner boundary of the INL (yellow arrowheads, Fig. 4A–F). Cells positive for β-galactosidase and Isl-1 in the GCL were RGCs and amacrine cells. Cells positive for Pax6 and β-galactosidase in the GCL and the inner boundary of the INL were ganglion and amacrine cells (Fig. 4G–I). However, little colocalization of β-galactosidase and Chx10 was observed in bipolar cells (Fig. 4J–L). Anti-Isl-1 also revealed no overlaps of Isl-1 and β-galactosidase in bipolar cells in the outer half of the INL (Fig. 4D–F). Anti-vimentin and anti-β-galactosidase showed no apparent colocalization in glial cells (Fig. 4M–O). The absence of β-galactosidase in bipolar, glial, and most photoreceptor cells in adult $math5^{Cre/+}$ $R26R^{lacZ/+}$ retina is likely due to the lack of *ROSA26* promoter activity in these cells (Fig. 2M, and data not shown). Whether bipolar, glial, and most photoreceptor cells arise from *math5* lineages remains to be examined. Nevertheless, our lineage analysis showed that *math5*-expressing cells gave rise to multiple retinal neuron types, demonstrating that Math5 expression alone in retina is not sufficient to drive the RGC differentiation pathway in progenitors. Other positive factors would be required in combination with *math5* to guide the proper differentiation of RGCs. Alternatively, negative factors in non-RGC cell lineages could function to antagonize *math5* and prevent their entry into the RGC differentiation pathway. Given its insufficient but indispensable role in the differentiation of RGCs, *math5* likely acts to define the RGC competence of retinal progenitors.

Postmitotic expression of math5-lacZ in developing mouse retinas

Though cell fate tracing experiments using lineage tracers in vertebrates have shown that all retinal neurons arise from a common pool of progenitors, the molecular mecha-

nisms governing the commitment of a progenitor to a given cell fate remain poorly understood, and it is unclear whether cell fate commitment occurs during or after the final mitosis (Turner and Cepko, 1987; Turner et al., 1990). The expression of *math5* in multiple retinal cell lineages indicates that *math5* is initially expressed in uncommitted retinal precursor cells. To further address the role of *math5* and to test whether retinal cell fate commitment occurs during or after the terminal mitosis, we next determined the timing of *math5* expression with respect to cell cycle during normal retinal development. Previous studies have shown that the retinal expression of *lacZ* in *math5-lacZ* knock-in mice faithfully recapitulates the expression of endogenous *math5* (Brown et al., 2001; Wang et al., 2001). The majority of the β-galactosidase-positive cells in the *math5-lacZ* retina showed a characteristic distribution in the ventricular zone at E11.5–E12.5. The E12.5 retinas from bromodeoxyuridine (BrdU)-treated $math5^{lacZ/+}$ embryos were double labeled with anti-β-galactosidase and anti-BrdU as S-phase marker or anti-Phospho-Histone H3 (Ser10) (PH3) as M-phase marker. As shown in Fig. 5A–C, most of the β-galactosidase-positive cells did not colocalize with BrdU-positive cells. Similarly, double labeling with anti-β-galactosidase and anti-PH3 revealed no colocalization of β-galactosidase-positive cells with PH3-positive M-phase cells (Fig. 5D–F). Therefore, our results demonstrate that Math5 is expressed in postmitotic cells. Combined with our prior findings that cells of *math5* origin give rise to multiple retinal neuron types, the postmitotic expression of *math5* further implies that retinal cell fate commitment occurs after the terminal mitosis.

Loss of Gfi1 expression in math5-null retina

In *Drosophila*, *ato* is required for the development of the first-born retinal neurons, R8 photoreceptors. Recent studies have shown that, immediately prior to R8 differentiation, *ato* expression is detected in a two- to three-cell cluster (Frankfort et al., 2001). These two- to three-cell clusters represent the final groups of cells that are fully competent to differentiate as R8 and only one R8 cell is derived from each competent cluster. The interaction of the negative regulatory factor Rough (Ro, a homeobox transcription factor) with the positive regulatory factors Ato and Senseless (Sens, a zinc-finger transcription factor) is essential to ensure that only one competent cell from each cluster is selected to become R8 and the rest form other photoreceptors such as R2-5 (Frankfort et al., 2001). Based on our lineage studies, we showed that, in addition to Math5, other positive and negative regulatory factors are required to determine the differentiation of RGCs in mice. It is therefore of interest to determine whether Sens-like factors could play similar positive regulatory roles in the development of mouse RGCs. In mice, the two *sens* homologues, *Gfi1* and *Gfi1b*, encode nuclear zinc-finger proteins. Previous studies have shown that *Gfi1* plays an essential role in regulating the differentiation of myeloid precursors into monocytes and granulocytes (Karsunky et al., 2002) and that *Gfi1b* is required for the development of erythroid and megakaryocytic lineages (Saleque et al., 2002). Recent experiments have demonstrated that neural expression of *Gfi1* is detected in the developing retina at E16.5–E18.5 and in the inner ear hair cells as well as in developing brain and dorsal root ganglion (Wallis et al., 2003). Targeted deletion of *Gfi1* results in the degeneration of inner ear hair cells in mice. To further analyze the expression of *Gfi1* and *Gfi1b* in retinal development and to define the genetic relationship of *Gfi1* and *Gfi1b* to *math5* and *brn-3b*, we analyzed the expression of *Gfi1* and *Gfi1b* in normal, *math5*-null, and *brn-3b*-null retinas by in situ hybridization. No detectable retinal expression of *Gfi1b* was observed from E11.5 to P0 (data not shown). In contrast to the retinal expression of *math5* in the ventricular zone, *Gfi1* expression was first detected at E14.5 and its expression was restricted in the GCL from E14.5 to E17.5 (Fig. 6A–C). After birth, *Gfi1* expression was undetectable in the retina (data not shown). The late onset and GCL-specific pattern of expression suggest that *Gfi1* could not act to maintain the expression of *math5* in the ventricular zone as *sens* positively regulates the expression of *ato* in R8 of *Drosophila*. To test whether the expression of *Gfi1* is dependent on the expression of *math5*, we investigated the expression of *Gfi1* in *math5*-null retinas at E15.5. At this stage, the expression of *Gfi1* mRNA was clearly detected in the GCL of control wild type retinas (Fig. 6B). However, its expression was drastically reduced in $math5^{lacZ/lacZ}$ retinas (Fig. 6D), demonstrating that *math5* acts upstream of *Gfi1* in RGC development. To further define the position of *Gfi1* in the *math5-brn-3b* genetic cascade, we then asked whether the expression of *Gfi1* was altered in *brn-3b*-null mutant retinas. As shown in Fig. 6E, compared with the control retina at E15.5 (Fig. 6B), the expression of *Gfi1* is downregulated in *brn-3b* mutant retinas, suggesting *Gfi1* as a downstream target of *brn-3b*.

Requirement for Math5 to activate a network of transcription factors implicated in sensory neurogenesis

Previous studies have shown that bHLH transcription factors often act in a genetic cascade during neurogenesis and myogenesis, and many important components of such cascades have been identified (Jan and Jan, 1993). In the development of sensory neurons, the expression of bHLH transcription factors, neurogenins (Ngn1 and Ngn2), precede the activation of other bHLH factors, such as NeuroD (Lee et al., 1995), Math3 (Takebayashi et al., 1997), Nscl-1, and Nscl-2 (Begley et al., 1992; Gobel et al., 1992). Targeted deletion experiments have shown that Ngn1 and Ngn2 are required for the expression of most of these bHLH factors (Fode et al., 1998; Ma et al., 1998). In addition, the Brn-3 family of POU-domain factors and the LIM homeodomain transcription factor, *Isl-1*, have been shown to be activated downstream of neurogenins (Anderson, 1999;

Perez et al., 1999). In order to identify the components of the *math5* pathway in RGC development, we then addressed whether the expression of these and other known RGC-associated transcription factors are dependent on *math5*. As shown in Fig. 7, the expression of *brn-3b, isl-1, isl-2, Nscl-1, Nscl-2,* and the POU-domain gene *RPF-1* (Zhou et al., 1996) were readily detected in the GCL of normal retinas at E14.5, and their expression was severely reduced in *math5*-null retinas, indicating their roles downstream of *math5* in the development of RGCs. Nevertheless, the expression of other transcription factor genes, including *pax6, chx10, NeuroD,* and *mash1,* was unaffected in *math5*-null retinas at E14.5 (data not shown). Previously, we have shown that *brn-3b* is essential for the terminal differentiation of RGCs (Gan et al., 1999). The roles of other transcription factors downstream of *math5* remain unknown in RGC development. However, the changes in the expression of these transcription factors in *math5*-null retinas suggest that the expression of *math5* is required to activate a comprehensive transcription network to determine the differentiation of RGCs.

Discussion

Postmitotic cell fate commitment of neuronal precursors in retina and role of math5 in determining the RGC competent state of progenitors

The competence model of retinal neurogenesis suggests that retinal progenitors undergo a series of competence states during retinal neurogenesis. At each competence state, the progenitors are competent to differentiate into a specific subset of retinal neurons in response to environmental cues (Cepko et al., 1996). Genetic studies with *math5, math3,* and *NeuroD* mutants have indicated that the competence states of progenitors are likely determined intrinsically by the expression of a combination of transcription factors and have confirmed the plasticity of the common retinal progenitors (Brown et al., 2001; Inoue et al., 2002a; Kay et al., 2001; Wang et al., 2001). However, it remains unknown when and how a progenitor commits to a certain retinal cell fate. Analysis of clones from retroviral infection reveals that two different cell types could be generated from the final cell division and leads to the conclusion that cell fate decisions occur during or after the final mitosis (Turner and Cepko, 1987; Turner et al., 1990). By employing the Cre-loxP strategy to study *math5* cell lineages, we show here that, during normal retinal development, cells of *math5* origin give rise to RGCs, amacrine, horizontal, and photoreceptor cells. Due to the lack of ROSA26 promoter activity in bipolar, glial, and most photoreceptor cells, we were unable to determine whether these cells are generated from *math5*-expressing cells. In order to sufficiently address whether Math5 is expressed in the precursors of these cells, it will require a conditional reporter gene that expresses in these cells. Nevertheless, the multiple retinal cell types of *math5* lineage suggest that *math5* expression alone is inadequate to determine the RGC cell fate and that cells expressing *math5* are not yet committed to a particular cell fate. Moreover, our results demonstrating postmitotic expression of *math5* strongly argue for occurrence of cell fate decisions after the final mitosis. Thus, retinal progenitors likely exit cell cycle to produce uncommitted neuronal precursors, and the precursors then undergo cell fate determination and cell differentiation when influenced by a combination of regulatory factors. Expression of *math5* endows the precursors with competence to differentiate into RGCs and the cell fate outcome of these precursors is later determined by additional positive and negative factors (Fig. 8A). In the absence of *math5,* precursors are incompetent to adopt the RGC fate. Rather, they choose non-RGC fate under the regulation of non-RGC differentiation factors.

Previous gain-of-function studies have shown that the forced expression of *ath5* homologues in developing *Xenopus* and chick retinas leads to the increased formation of RGCs at the expense of amacrine, bipolar, and Muller cells, suggesting that *ath5* expression is sufficient for the formation of RGCs (Kanekar et al., 1997; Liu et al., 2001). Nevertheless, careful examination of these results has indicated that the forced expression of *ath5* in developing retina fails to convert a majority of *ath5*-misexpressing cells into RGCs. Misexpression of *Xath5* in 50–100% of retinal progenitors by lipofection of optic vesicles or by microinjection of blastomere D.1.1 in *Xenopus* leads to an approximate two-to three-fold increase of RGCs (Kanekar et al., 1997). Overexpression of *cath5* and *math5* in developing chick retina infected with RCAS-*cath5* and RCAS-*math5* retroviruses showed that, while exogenous *cath5* and *math5* are highly expressed in greater than 50% of all retinal cells, the forced expression only results in a 30% increase in the number of RGCs as detected by RGC marker neurofilament 200 (Liu et al., 2001). Since RGCs represent less than 5% of total retinal cells in developing retina, the above studies indicate that misexpression of *ath5* in a majority of retinal pregenitors is insufficient to convert them into RGCs. The misexpression studies of *ath5* are largely consistent with our cell lineage results, showing that *math5* expression alone is insufficient to determine RGC cell fate during normal retinal development and that additional factors are needed to guide the uncommitted *math5*-expressing cells positively into the RGC differentiation pathway or negatively into a non-RGC pathway. One likely explanation for the limited increase of RGC formation in *ath5*-transfected retinas is that the prolonged expression of *ath5* in progenitors from viral and constitutive promoters increases the number of RGC-competent precursors and enables the precursors to remain RGC competent for an extended period. Thus, more RGCs are generated. Alternatively, compared with the transient expression of endogenous *ath5,* the lasting, high level expression of exogenous *ath5* could overcome the negative

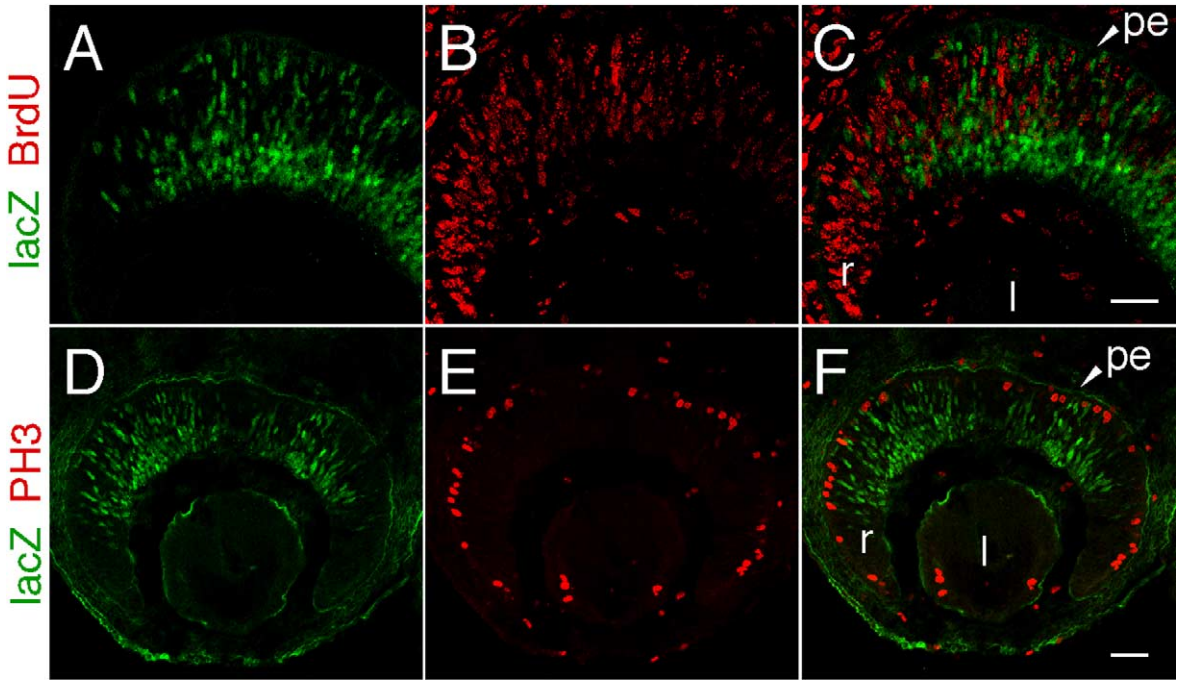

Fig. 5. Postmitotic expression of *math5* in the developing retina. Heterozygous *math5*$^{lacZ/+}$ retinas at E12.5 were prepared for double immunolabeling with anti-β-galactosidase and cell cycle markers. (A, D) Anti-β-galactosidase (green) labeling reveals the expression of nuclear Math5-lacZ in cells across the retina. (B) Anti-BrdU (red) shows the nuclei of proliferating cells at S-phase. (C) Merged image of (A) and (B). (E) Anti-Phospho-Histone 3 (PH3) (red) marks the nuclei of proliferating retina cells. (F) Merged image of (D) and (E). r, retina; l, lens; pe, pigmented epithelium. Scale bar, 100 μm.

regulation in the non-RGC pathway and lead to an increase in RGC differentiation.

In an attempt to identify potential positive factors for RGC differentiation, we have shown that Math5 activates the expression of a transcription factor network that includes Brn-3b. In our previous studies, we have shown that

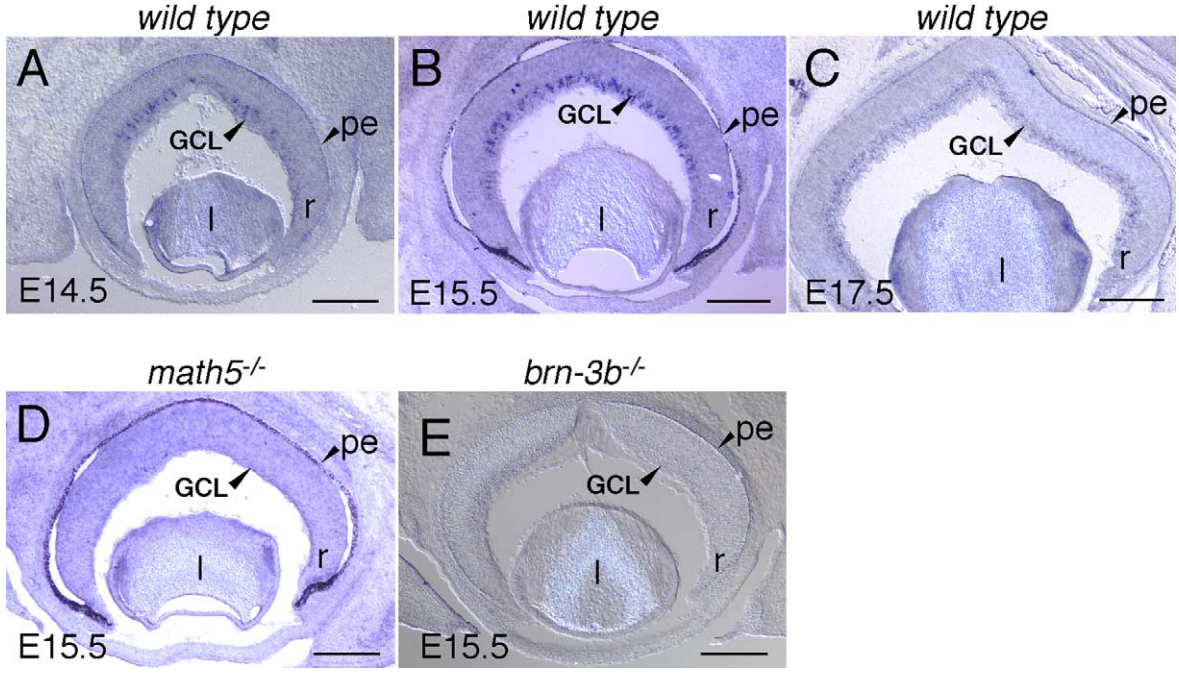

Fig. 6. Downregulation of *Gfi1* expression in *math5* and *brn-3b* mutant retinas. In situ hybridization of wild type retinal sections with Gfi1 probe at E14.5 (A), E15.5 (B), and E17.5 (C). (D) Reduced expression of Gfi1 in *math5*-null retina at E15.5. (E) Reduced expression of Gfi1 in *brn-3b*-null retina at E14.5. r, retina; l, lens; pe, pigmented epithelium; GCL, ganglion cell layer. Scale bar, 100 μm.

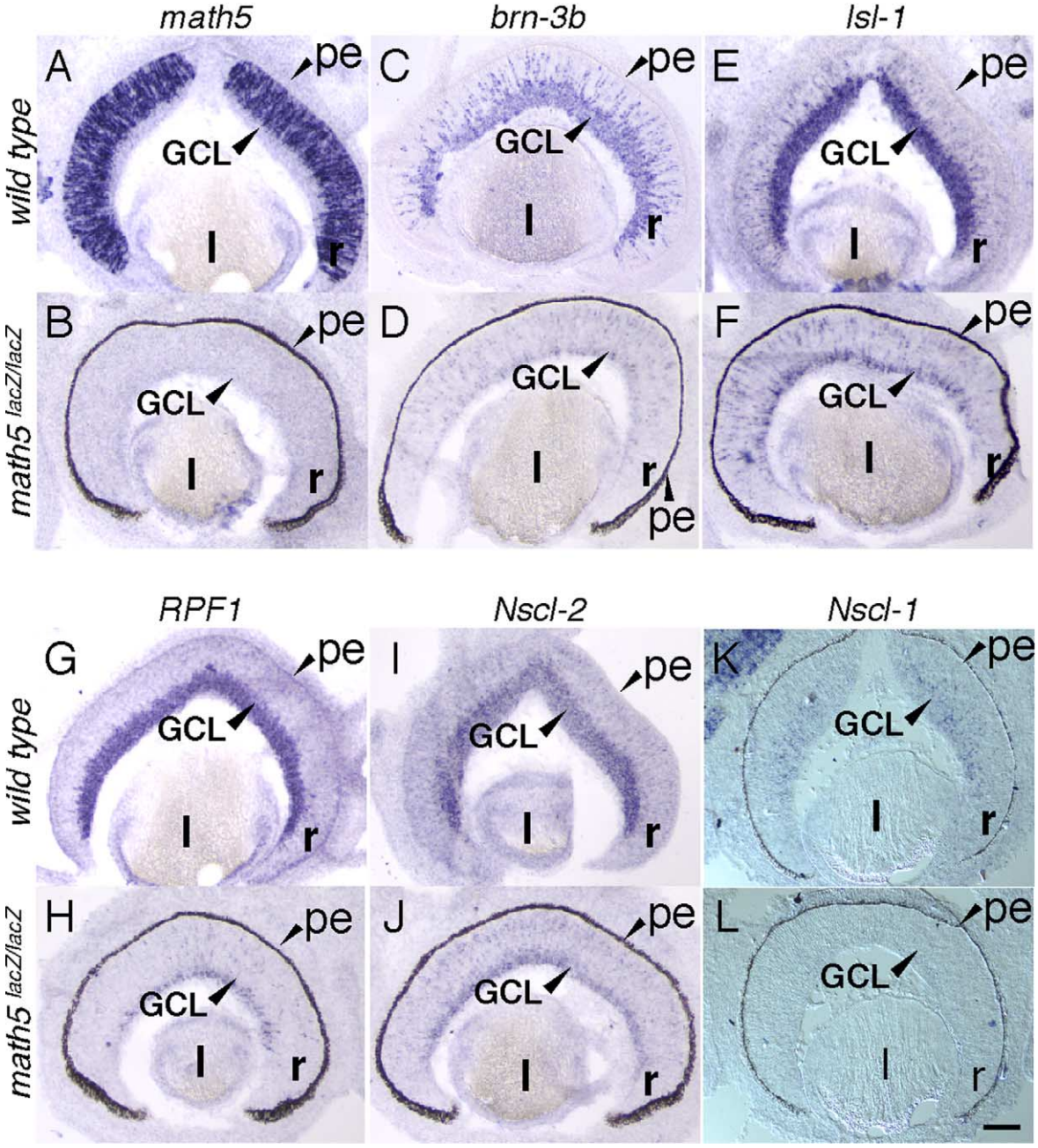

Fig. 7. Requirement for *math5* to activate a network of transcription factors. Section *in situ* hybridization analyses of wild type (top) and *math5*-null (bottom) retinas at E14.5 show that the RGC expression of *brn-3b* (C, D), *isl-1* (E, F), *RPF1* (G, H), *Nscl-2* (I, J), and *Nscl-1* (K, L) are dependent on Math5 activity. (A, B) *math5* probe as controls. r, retina; 1, lens; pe, pigmented epithelium; GCL, ganglion cell layer. Scale bar, 100 μm.

brn-3b is essential for the later events of RGC differentiation, including axonal growth, pathfinding, and survival of developing RGCs, but not for the initial differentiation and migration of RGCs (Gan et al., 1996, 1999). The roles of other *math5* downstream factors in RGC development are currently unknown. If *math5* is required to determine the RGC competence and to initiate the RGC differentiation program and other factors confine the RGC differentiation pathway, it would be interesting to test for synergistic effects of coexpressing *math5* and its downstream genes in retinal progenitors.

Incomplete conservation of retinal development pathways in Drosophila and mice

The loss-of-function and cell lineage studies of *math5* suggest a remarkable similarity in the role of *math5* and *ato* in the neurogenesis of mouse and fly retinas. In fly retinal

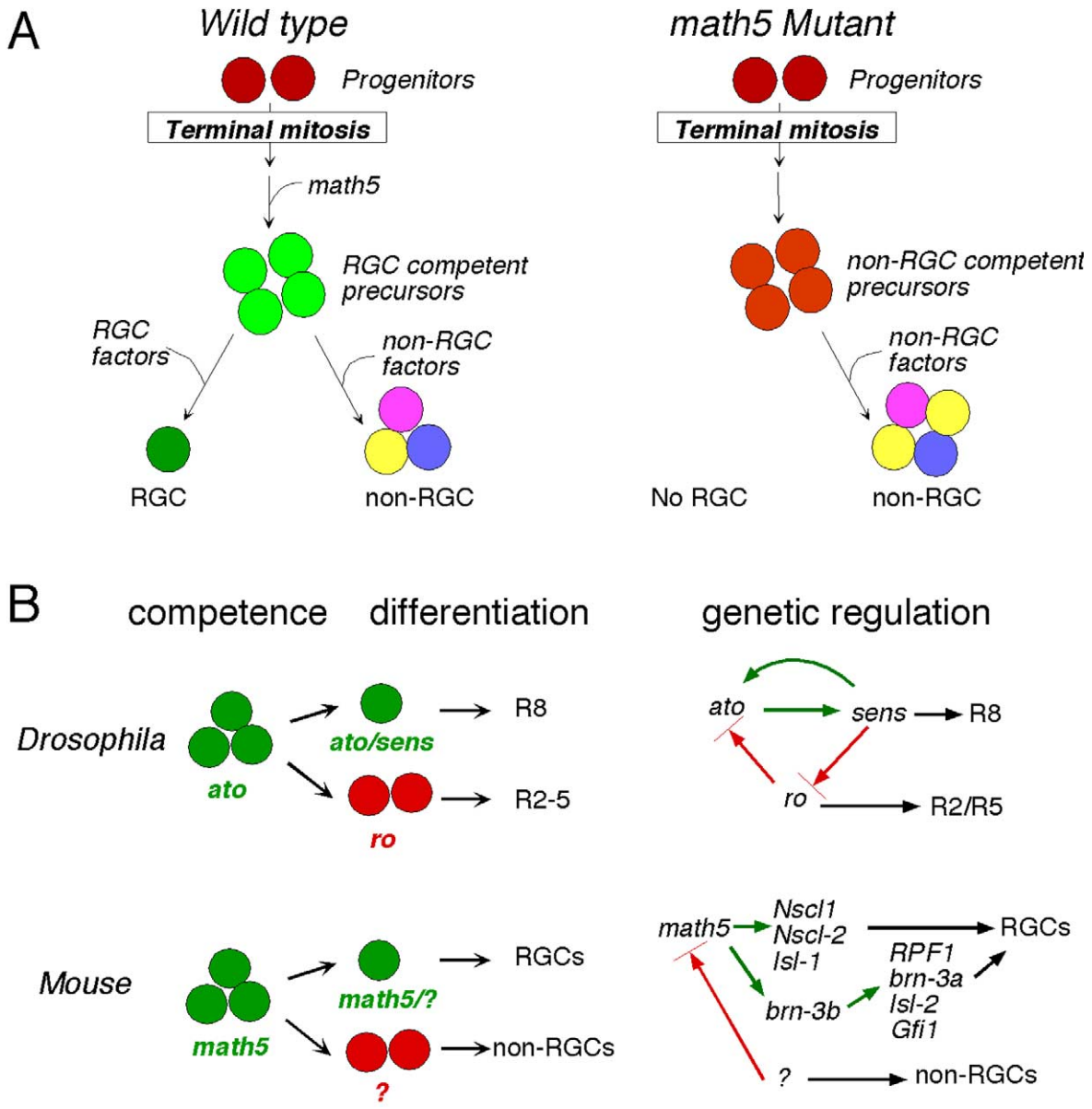

Fig. 8. Models of retinal neuron differentiation. (A) A model of *math5* function during retinal differentiation. In wild type, retinal progenitors exit the cell cycle to generate uncommitted precursors and the expression of *math5* acts to make the precursors RGC-competent. Some precursors become RGCs under the regulation of RGC differentiation factors. The remaining precursors choose non-RGC fates in the presence of non-RGC regulators. In *math5* mutant, precursors are not RGC competent and no RGCs are generated. All precursors adopt non-RGC fates and the presence of excess precursors leads to the formation of excess non-RGC neurons. (B) Comparison of the role of *ato* and *math5* in determining the competence of retinal progenitors and the potential transcription networks and cascades regulating the development of R8 in *Drosophila* and ganglion cell in the mouse retina. Arrows in green and red indicate positive and negative regulation, respectively. The arrows indicate the position of transcription factors in the *math5-brn-3b* regulatory hierarchy, not necessarily the direct transcriptional regulation. The regulatory relationship among *math5, Nscl-1, Nscl-2, RPF1, brn-3b, isl-1, isl-2,* and *Gfi1* is identified in this study and those among *brn-3b, brn-3a, RPF1,* and *isl-2* are from other studies (Z.Y. and L.G. unpublished observations).

development (Fig. 8B), *ato* is initially widely expressed and its expression begins to become restricted in the morphogenetic furrow (MF) (Jarman et al., 1995). In the MF, *ato* is limited to a small group of two to three cells which form the R8 equivalence group (Hsiung and Moses, 2002), and later, to a single cell, which becomes the R8 photoreceptor. Recent studies have shown that the restricted expression of *ato* and the formation of R8 is regulated by Sens and Ro (Frankfort et al., 2001). As shown in Fig. 8B, *sens* is downstream of *ato* and acts to maintain *ato* expression in R8 equivalence groups and R8 cells, and to ensure proper differentiation of R8. On the other hand, the expression of *ro* is mutually exclusive with that of *ato*. Within the MF, *ro* is expressed in all cells but the R8 equivalence group. Later, *ro* is expressed in developing R2, R3, R4, and R5 to suppress R8 fate and to enable the cells to adopt R2/R5 fates (Dokucu et al., 1996). Genetic evidence indicates that *sens* acts to represses *ro* in R8. In *sens* mutant ommatidia, the

presumptive R8 cell expresses *ro* and chooses R2/R5 fate. Thus, *ato* activates *sens* in the R8 equivalence group and R8, and *sens* in turn acts to maintain *ato* expression and R8 differentiation. Sens plays this role by preventing *ro*-mediated repression of R8 and induction of R2/R5 fate.

In mouse, the formation of the first born RGCs requires the function of *math5* (Brown et al., 2001; Wang et al., 2001). In this study, we show that *math5*-expressing cells develop into ganglion, amacrine, horizontal, and photoreceptor cells, demonstrating that *math5* expression alone is insufficient to confer RGC differentiation during normal retinal neurogenesis. Our results imply that, like *ato*, *math5* is initially expressed to determine the RGC competence of progenitors (Fig. 8B) and, in combination with other positive factors, drives RGC differentiation. We have shown by in situ hybridization that *Gfi1* is expressed in developing RGCs. By comparing its expression in wild type and *math5* mutant retinas, we have shown that *Gfi1* is a downstream gene of *math5*. However, unlike *sens* in *Drosophila*, *Gfi1* is expressed in ganglion cells after *math5* expression is ceased, and the loss of *Gfi1* expression in *brn-3b* mutant retina indicates that *Gfi1* is further downstream of *brn-3b*. Our results imply that, in mice, *Gfi1* is unlikely to upregulate *math5*. Rather, the late expression of *Gfi1* could be associated with the terminal differentiation of RGCs. Interestingly, our findings of the relationship of *math5* and *Gfi1* in RGC development closely resemble those of *math1*, an *ato* homologue closely related to *math5*, and *Gfi1* in the development of inner ear sensory hair cells (Wallis et al., 2003). Previous studies have shown that, like *math5*, *math1* expression is postmitotic in cells of the inner ear sensory epithelia (Chen et al., 2002). Mice null for *math1* fail to generate both cochlear and vestibular hair cells (Bermingham et al., 1999). *Gfi1*, as a downstream gene of *math1*, is known to be essential for survival and terminal differentiation but not for the cell fate specification of inner ear hair cells (Wallis et al., 2003). Moreover, we and others have shown that *brn-3c*, a member of the highly conserved *brn-3* POU-domain gene family, is also expressed and required for the differentiation and survival of inner ear hair cells (Erkman et al., 1996; Xiang et al., 1997). The regulatory relationship of *math1*, *brn-3c*, and *Gfi1* is not fully determined yet. Nevertheless, compared with the *math5-brn-3b-Gfi1* regulatory pathway in RGC development, a similar transcription hierarchy of *math1-brn-3c-Gfi1* is likely to be conserved in the development of inner ear, with *math1* determining the hair cell competence of inner ear progenitors and *brn-3c* and *Gfi1* regulating the terminal differentiation and survival of the hair cells.

Similarly, the increased number of amacrine and cone cells in *math5* mutant retinas suggests that *math5* expression negatively regulates the differentiation of non-RGC neurons and/or that other negative regulatory factors exist to promote non-RGC fate (Fig. 8B). Whether Ro-like factors play such a role in mouse retina remains unclear as we are yet to identify *ro* homologues in mice. Recent findings show that loss of *NeuroD* and *math3* leads to upregulation of *math5* and a concurrent cell fate switch from amacine to ganglion cells (Inoue et al., 2002a). Other bHLH factors have also been shown to regulate retinal cell fate choices (Hatakeyama et al., 2001; Morrow et al., 1999). Thus, it is possible that bHLH factors could act in a mutual suppressive mechanism to determine retinal cell fate.

Acknowledgments

We thank Drs. William Klein, Roman Giger, Douglas Portman, and Joe Miano for critical reading of the manuscript and helpful discussions, and Drs. Qiang Li and Feng Liang for experimental assistance. This work is supported by grants from The Rosanne H. Silbermann Foundation and National Institutes of Health (EY13426). L.G. is a Rosanne H. Silbermann Scholar.

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